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Opposite Effects of Nitric Oxide on Identified Inhibitory and Excitatory Cholinergic Synapses of Aplysia Californica

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Summary

The effects of nitric oxide on evoked acetylcholine (ACh) release were studied at two identified cholinergic neuro-neuronal synapses of the nervous system of the mollusc *Aplysia californica*. The NO-donor, 3-morpholinosydnonimine (SIN-1), decreased the amplitude of evoked inhibitory postsynaptic currents (buccal ganglion) and potentiated that of evoked excitatory postsynaptic currents (abdominal ganglion). SIN-1 acted by modulating the number of ACh quanta released. 8Br-cGMP mimicked the effects of NO on ACh release in both types of synapses thus pointing to the involvement of a NO-sensitive guanylate cyclase. Presynaptic voltage-dependent Ca^{2+} and K^+ (I_A and late outward rectifier) currents were not modified by SIN-1 suggesting another final target for NO/cGMP. The labelling of a NO-synthase by immunostaining in several neurones as well as the modulation of ACh release by L-arginine indicate that an endogenous NO-synthase is involved in the modulation of synaptic efficacy in both buccal and abdominal ganglia.

Key words
Nitric oxide – Guanylate cyclase – Synaptic transmission – Voltage-gated presynaptic currents

Introduction

Nitric oxide, a free radical gas formed endogenously by several vertebrate cell types (Moncada *et al.* 1991) and probably by invertebrates (Jacklett and Gruhn 1994, Moroz *et al.* 1994), has been implicated as a diffusible intercellular messenger subserving use-dependent modifications of synaptic efficacy such as long-term potentiation and long-term depression in the central nervous system (Schuman and Madison 1994).

At the level of cholinergic transmission in vertebrate preparations, NO has been shown to exert a potentiating action at chick ciliary ganglia (Lin and Bennett 1994), in the basal forebrain of rat (Prast and Philippu 1992), in the rat striatum (Guevara-Guzman *et al.* 1994) as well as in neuroglioma PC-12 cells and in brain synaptosomes (Hirsh *et al.* 1993). NO has also a depressive action on ACh release as shown in guinea-pig myenteric plexus (Wiklund *et al.* 1993, Kilbinger and Wolf 1994). It thus appears that NO can produce opposite effects on cholinergic synapses efficacy according to the preparation although all these effects might be cGMP-dependent. It is generally agreed that NO increases cGMP levels through the activation of the cytosolic form of guanylate cyclase. But the molecular target(s) on which the NO/cGMP pathway acts to modify transmitter release still remain unknown.

In the present study, we have focused on the mechanisms by which NO could modulate synaptic efficacy at two identified cholinergic neuro-neuronal synapses in the buccal ("inhibitory synapse") and the abdominal ("excitatory synapse") ganglia of *Aplysia californica*. For this purpose, we have studied the presynaptic effects of the exogenous NO-donor, 3-morpholinosydnonimine, and of L-arginine, the substrate of NO-synthase, on transmitter release by measuring the number of evoked quanta released and we investigated a possible action of NO on presynaptic voltage-gated Ca^{2+} and K^+ channels. In addition, using specific antibodies, we have sought to reveal the presence of a neuronal NO-synthase.

Materials and Methods

All experiments were performed on buccal and abdominal nervous ganglia of *Aplysia californica* adult specimens (Marinus Inc., Long Beach, California).

Electrophysiology

The desheathed ganglia were maintained at 22 °C and continuously superfused with artificial sea water containing NaCl, 460 mM; KCl, 10 mM; $CaCl_2$, 11 mM; $MgCl_2$, 25 mM; $MgSO_4$, 28 mM; Tris-HCl buffer pH 7.8, 10 mM to which drugs were added as appropriate. We used two preparations: the cholinergic "H-type" Cl^--dependent inhibitory synapse in the buccal ganglion between B_4 or B_5 presynaptic neurone and B_3 or B_6 postsynaptic neurone and a "D-type" cationic Na^+-dependent cholinergic excitatory synapse on the R15 abdominal neurone activated by threshold stimulation of the right pleuro-abdominal connective (Gerschenfeld *et al.* 1967). Because of the low resistance of the electrodes used, Cl^- leakage from KCl electrodes could result in shifts of the E_{Cl^-} towards less negative voltages due to changes in intracellular Cl^- concentration. This problem was accounted for in our recordings by regularly readjusting the reversal potential for postsynaptic responses. Therefore, the postsynaptic response was recorded as a current *(I)* and expressed as a conductance *(G)* by means of the following equation, $G = I / V - V_{eq}$ where V is the holding potential (−80 mV or −70 mV) and V_{eq} the equilibrium potential for Cl^- ions. The quantal analysis of transmitter release was performed in the inhibitory synapse using the described method of long-duration induced postsynaptic current (Baux *et al.* 1990, Fossier *et al.* 1990, 1992) which allows the measurement of quantal evoked ACh release in the absence of spike generation.

Presynaptic Ca^{2+} currents were elicited by 50 ms depolarizing steps to a variety of test potentials from a holding potential of −50 mV (Trudeau *et al.* 1993). The bath solution contained tetrodotoxin (100 μM), tetraethylammonium (50 mM) and 4-aminopyridine (4 mM) to eliminate Na^+ and K^+ contamination of the Ca^{2+} current. The extracellular Ca^{2+} concentration was raised to 55 mM. I/V curves were leakage subtracted. The early (I_A) and delayed rectifier (I_K) potassium currents were elicited by 100 ms depolarizing steps from a holding potential of −50 mV in artificial sea water containing 100 μM tetrodotoxin.

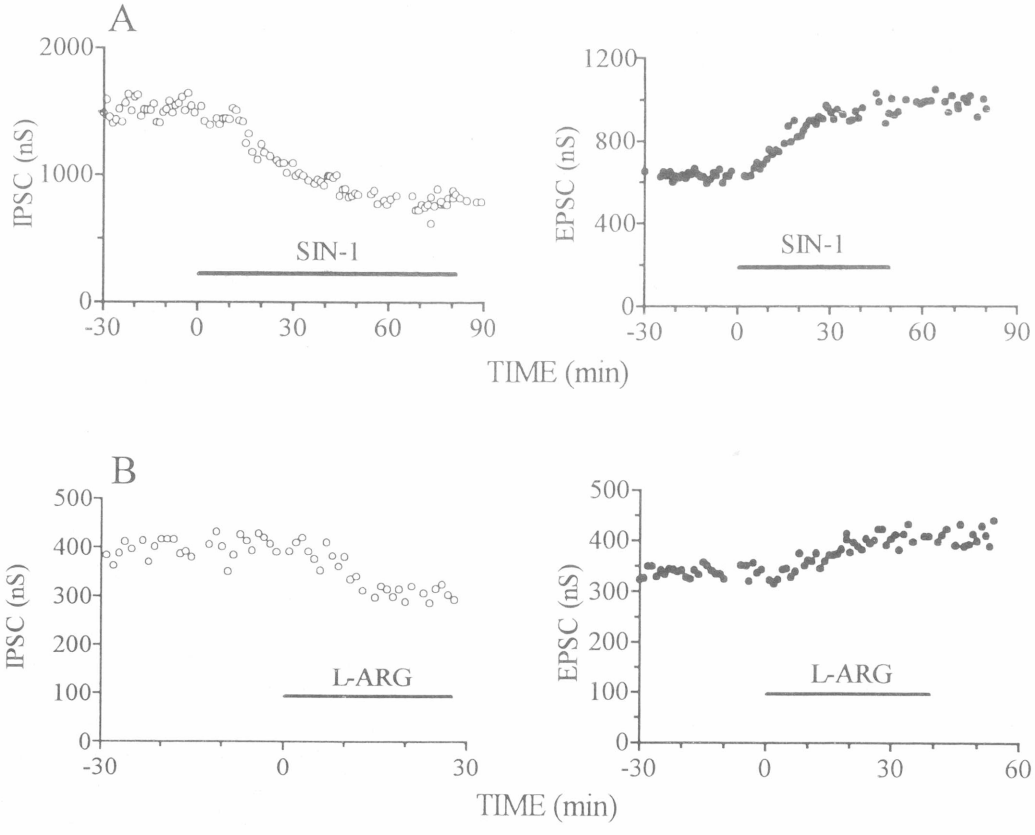

Fig. 1

Opposite effects of A) exogenous NO (NO-donor: SIN-1, 100 μM), and B) endogenous NO (L-arginine, L-ARG, as substrate of NO-synthase) on the inhibitory (IPSC) (left column) and excitatory (EPSC) (right column) postsynaptic currents in the buccal and abdominal ganglia, respectively.

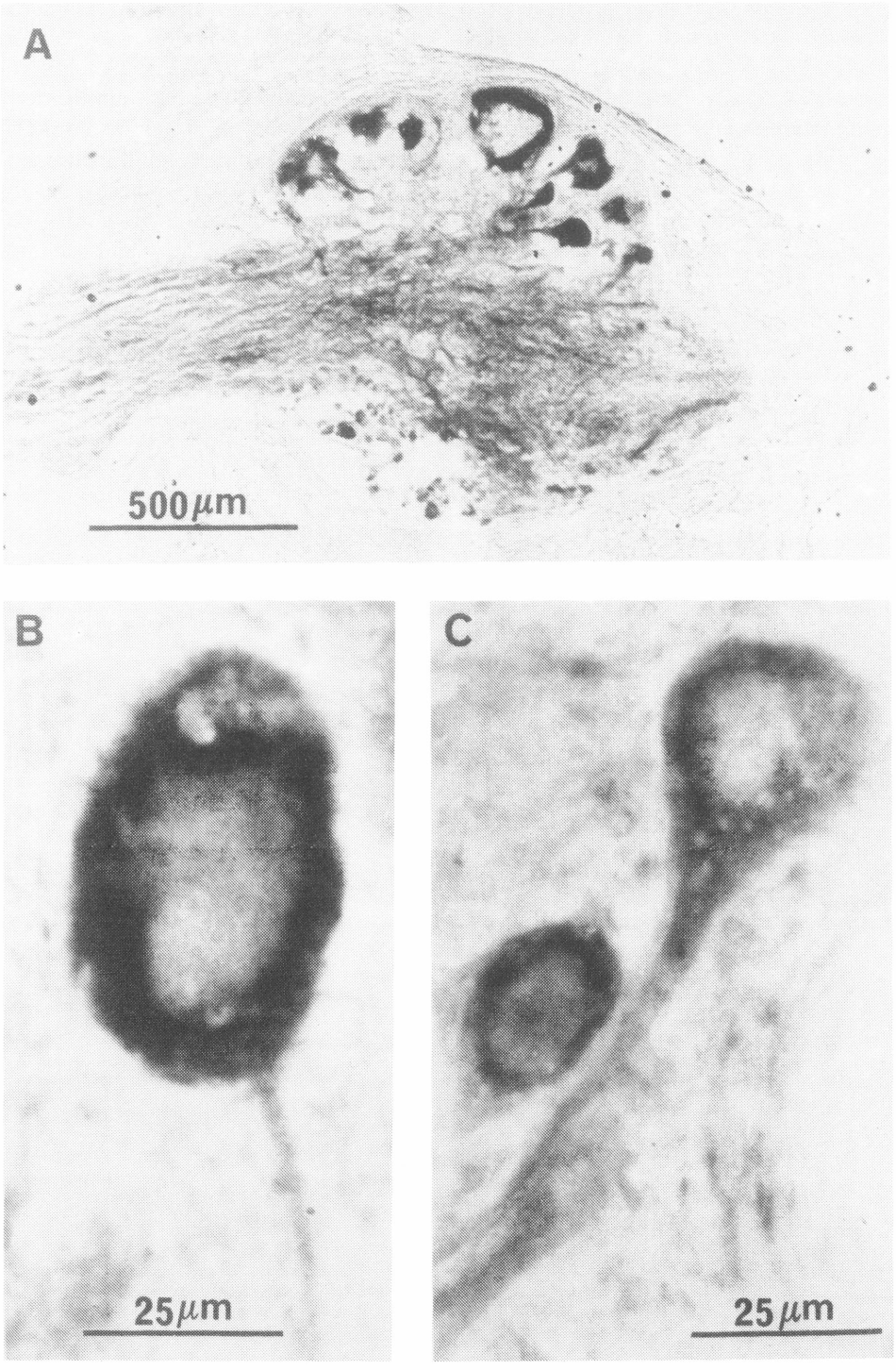

Fig. 2
Immunostaining of the neuronal NO-synthase. A) buccal ganglion exhibits positive large and small cell bodies as well as processes in the neuropile. Higher magnification of positive neurones in the abdominal (B) and buccal (C) ganglia indicates a cytoplasmic localization of NO-synthase.

Histology

Serial sections were prepared as previously reported (Meulemans *et al.* 1995). Immunocytochemistry for NO-synthase (NOS) was performed with a polyclonal rabbit antibody raised against the C-terminal 1415-1429 amino-acid sequence of rat cerebellar NOS (Alm *et al.* 1993). Ganglion sections were dried for 15 min and then incubated in Tris-HCl buffer salt pH 7.6 containing 10 % horse serum and 0.3 % triton X-100 for 60 min at room temperature. After rinsing in Tris-HCl buffer salt, sections were incubated with the NOS antiserum (1:2000) in a moist chamber at +4 °C overnight. The sections were then

washed in tris-HCl buffer salt and incubated with biotinylated donkey anti-rabbit immunoglobulins (1:2000, Jackson Immunoresearch Laboratories Inc) in a moist chamber at +4 °C overnight. After thorough washing, sections were exposed to streptavidin-horseradish peroxidase (1:1000, streptavidin-POD conjugate, Boehringer) for 90 min. Staining was visualized by 0.02 % diaminobenzidine as chromogen (with nickel intensification) plus H_2O_2 at a final concentration of 0.0015 %.

Drugs

3-morpholinosydnonimine hydrochloride (SIN-1) was purchased from Biomol. L-Arginine and 8Br-cGMP were obtained from Sigma.

Results

The NO-donor, SIN-1 (100 μM), decreased the amplitude of the inhibitory postsynaptic current (IPSC) evoked by a presynaptic action potential, by 40–50 % (Fig. 1A left graph) whereas it potentiated the amplitude of the excitatory postsynaptic current (EPSC) by 50–70 % (Fig. 1A right graph) after 30–40 min application. The bath application of the substrate of NO-synthase, L-arginine (1 mM) induced, after a short delay, similar modifications of postsynaptic responses (Fig. 1B). These results point to a modulatory role of endogenous NO on synaptic efficacy at both inhibitory and excitatory synapses. The next point attempted was to reveal the presence of NO-synthase in ganglion neurones.

NO-synthase immunoreactivity appeared in the cytoplasm of both small and large neurones in buccal (Fig. 2A,C) and abdominal (Fig. 2B) ganglia. Intensely stained fibres were also visualized in connectives and in the proximal part of the peripheral nerves in both ganglia (Fig. 2A). NADPH-diaphorase staining labelled the same neurones (not shown). The co-existence of NADPH-diaphorase activity and NO-synthase immunoreactivity argues for NO-synthase activity in *Aplysia ganglia*.

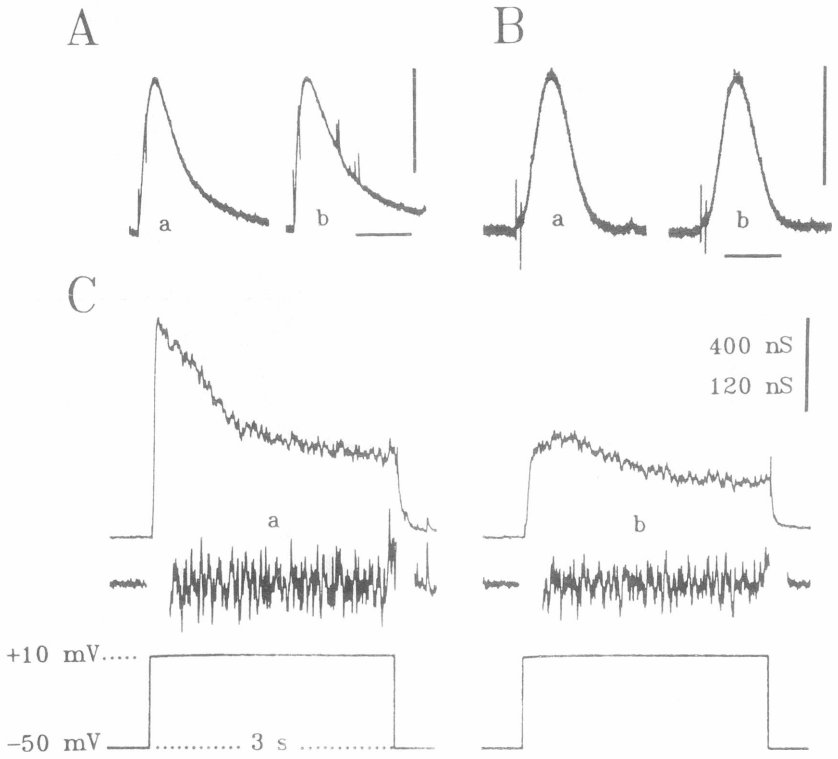

Fig. 3

Presynaptic action of NO. The amplitude of the postsynaptic responses to ionophoretic ACh application was unmodified in the presence of SIN-1 (100 μM) in the buccal (A) and abdominal (B) ganglia (horizontal bars : 2 s). C) In the buccal ganglion, the amplitude of the postsynaptic response (upper traces) induced by a long depolarization (3 s) of the voltage-clamped presynaptic neurone (bottom traces) was decreased by SIN-1. The middle trace represents the high frequency component of the postsynaptic current. a, control; b, after 40 min SIN-1. Statistical analysis of this postsynaptic response permits to calculate the mean amplitude and the mean decay time of miniature postsynaptic currents which sum to build up this response. Because the amplitude of the calculated evoked miniature in this preparation decreases when the mean amplitude of the postsynaptic response increases (Baux and Tauc 1987), we compare normalized miniatures at a given postsynaptic response amplitude. The amplitude of the calculated miniature was 0.42 nS (at a 400 nS postsynaptic response) and was unchanged in the presence of SIN-1. The mean decay time of the miniatures also remained constant (16 ms).

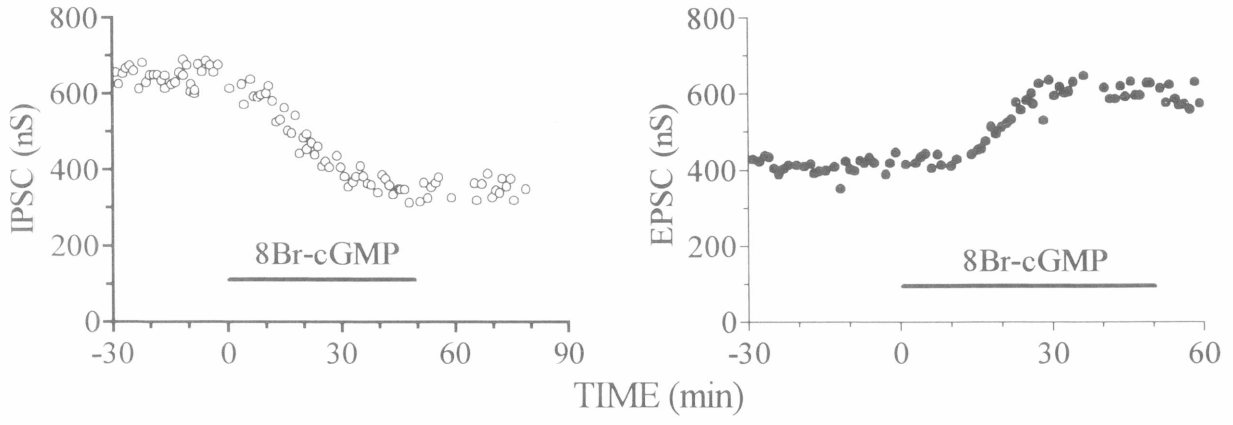

Fig. 4
8Br-cGMP (1 mM) mimicked the effects of SIN-1 on both IPSCs (left graph) and EPSCs (right graph).

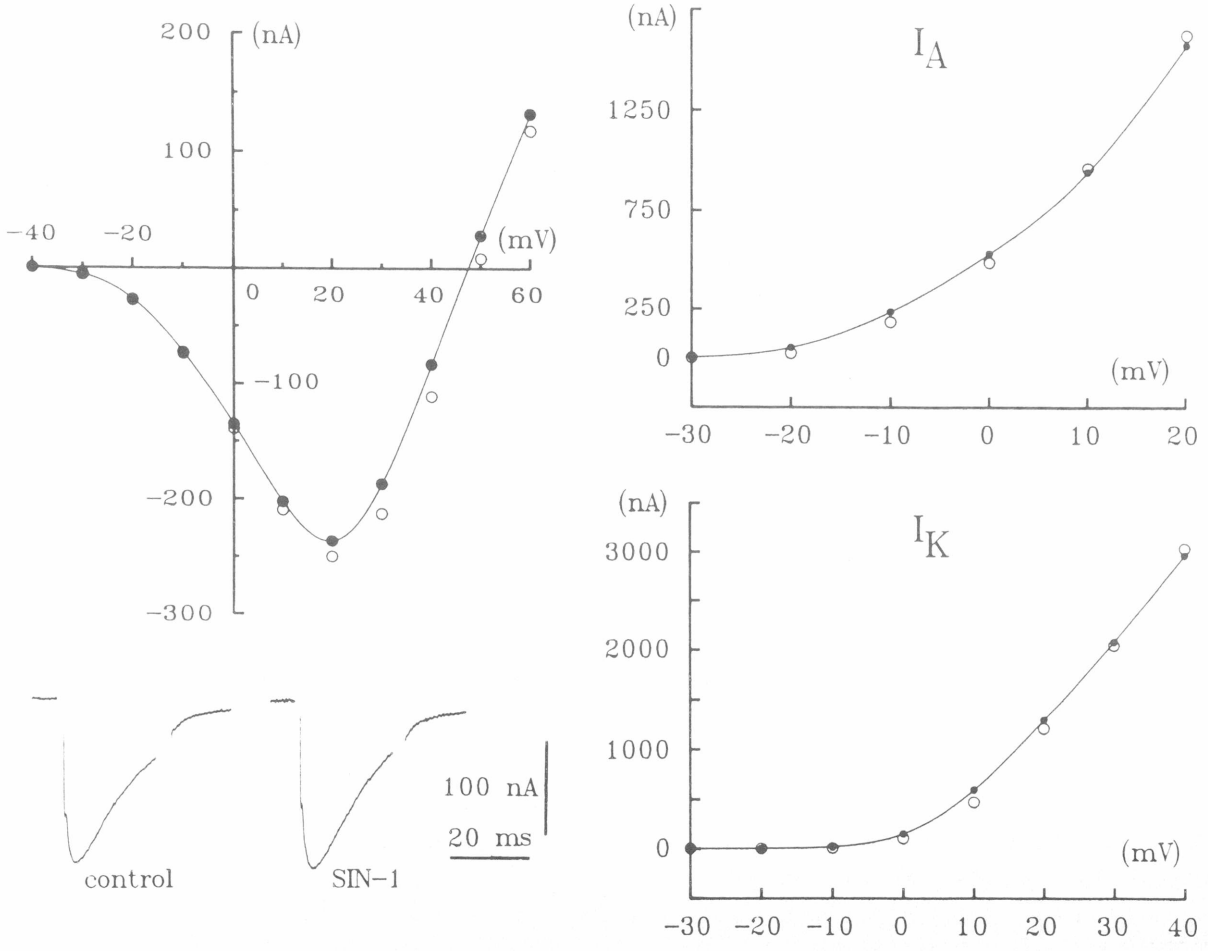

Fig. 5
I/V curves for Ca^{2+} and K^+ currents. Application of SIN-1 (100 μM) for 1 hour did not change the presynaptic Ca^{2+} current (left graph). Recordings represent the peak Ca^{2+} current before and after SIN-1 application. Presynaptic I_A and I_K potassium currents (right graphs) were unchanged after SIN-1 application for 1 hour (full circle – control; open circle – SIN-1).

NO could act either at the pre- or postsynaptic level to modulate synaptic transmission. The amplitude of postsynaptic responses to ionophoretic application of ACh was not modified in the presence of SIN-1 (Fig. 3A,B) indicating that the action of NO was presynaptic. Statistical analysis of the postsynaptic response evoked by long-duration depolarization of the voltage-clamped presynaptic neurone in the inhibitory synapse (Fig. 3C) showed that NO modulates synaptic transmission not by affecting the presynaptic spike but by modifying the number of quanta released by the presynaptic terminal. The mean amplitude and decay time of the calculated evoked miniatures which sum to build up the postsynaptic responses evoked by depolarization of the presynaptic neurone were unmodified (see legend of Figure 3) confirming that NO had no postsynaptic effect.

The cell-permeant and non-hydrolysable analog of cGMP, 8Br-cGMP, applied in 1 mM concentration, decreased the amplitude of IPSCs (Fig. 4, left graph) with a time course and an extent similar to that elicited by SIN-1. 8Br-cGMP had an opposite effect on the amplitude of EPSCs (Fig. 4, right graph). Statistical analysis of long duration-induced postsynaptic currents showed that the decrease of the inhibitory postsynaptic response by 8Br-cGMP was due, as for SIN-1, to a reduction in the number of evoked released quanta (not shown). As 8Br-cGMP mimics the modulatory effects of NO, we concluded that NO probably activates guanylate cyclase at both synapses.

The presynaptic terminal of the inhibitory synapse is easily maintained under voltage-clamp conditions and this allowed us to measure presynaptic voltage-gated Ca^{2+} and K^+ currents in order to check whether they are the final target of the NO/cGMP pathway. The application of SIN-1 (100 μM) did not induce any change of the presynaptic Ca^{2+} current, even after more than 1 hour of application (Fig. 5, left I/V curve). In the same way, the fast outward K^+ current, I_A and the late rectifier K^+ current, I_K (Fig. 5) were unchanged.

Discussion

In this study we demonstrated that NO has opposite presynaptic effects on cholinergic transmission when it modulates an inhibitory or an excitatory synapse. This is in agreement with scarce observations on different preparations showing that NO could increase (Prast and Philippu 1992, Guevara-Guzman *et al.* 1994, Lin and Bennett 1994) or decrease (Wiklund *et al.* 1993, Kilbinger and Wolf 1994) ACh release. Nevertheless, an opposite action of NO on cholinergic synapses is shown here for the first time in the same central nervous system. This opposite control of NO on ACh release at different synapses may be due to an action on different downstream mechanisms involved in the neurotransmitter release machinery.

NO has been reported to change K^+ conductance in smooth muscle (Archer *et al.* 1994) and in cultured neurones of avian ciliary ganglia (Cetiner and Bennett 1993), or Ca^{2+} conductances of L-type channels in frog ventricular myocytes (Mery *et al.* 1993) and of L- and N-type channels in PC12 cells (Desole *et al.* 1994). By contrast, in our preparations, NO did not change the current flowing through presynaptic K^+ or Ca^{2+} channels (Fig. 5).

The finding that NO can modulate quantal ACh release in opposite ways without affecting Ca^{2+} and K^+ fluxes raises the question of the synaptic target of the NO/cGMP pathway. One possibility would be an action of cGMP on Ca^{2+} stores. Such a possibility was proposed in macrophages (Randriamanpita *et al.* 1991) and in sea urchin eggs (Galione *et al.* 1993). The contribution of Ca^{2+} stores in the control of the concentration of Ca^{2+} in the terminal could effectively play an important role in triggering ACh release (Fossier *et al.* 1992). Another possibility could be that, besides the activation of cGMP pathway, NO acts directly on presynaptic proteins implicated in neurotransmitter release (Hess *et al.* 1993) and/or enhances the activity of ADP-ribosyl transferase which might account, for instance, for the NO-induced long-term potentiation in the hippocampus (Schuman *et al.* 1994).

The presence of NO-synthase in the neurones and the non-ambiguous modulation of ACh release by L-arginine give the opportunity to study the presynaptic mechanism(s) regulated by NO on a very convenient model.

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